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Phytochemistry 66 (2005) 153–163
PHYTOCHEMISTRY
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Purification and properties of a β-galactosidase from carambola fruit with significant activity towards cell wall polysaccharides

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Received 1 July 2004; received in revised form 9 November 2004
Available online 19 December 2004

Abstract

β-Galactosidase (EC. 3.2.1.23) from ripe carambola (*Averrhoa carambola* L. cv. B10) fruit was fractionated through a combination of ion exchange and gel filtration chromatography into four isoforms, viz. β-galactosidase I, II, III and IV. This β-galactosidases had apparent native molecular masses of 84, 77, 58 and 130 kDa, respectively. β-Galactosidase I, the predominant isoform, was purified to electrophoretic homogeneity; analysis of the protein by SDS–PAGE revealed two subunits with molecular masses of 48 and 36 kDa. N-terminal amino acid sequence of the respective polypeptides shared high similarities albeit at different domains, with the deduced amino acid sequence of certain plant β-galactosidases, thus, explaining the observed low similarity between the two subunits. β-Galactosidase I was probably a heterodimer that have glycoprotein properties and a p*I* value of 7.2, with one of the potential glycosylation sites appeared to reside within the 48-kDa-polypeptide. The purified β-galactosidase I was substantially active in hydrolyzing (1 → 4)β-linked spruce and a mixture of (1 → 3)β- and (1 → 6)β-linked gum arabic galactans. This isoform also had the capability to solubilize and depolymerize structurally intact pectins as well as to modify alkaline-soluble hemicelluloses, reflecting in part changes that occur during ripening.

Keywords: *Averrhoa carambola* L.; Oxalidaceae; Fruit; β-Galactosidase; Enzyme purification and properties; Cell wall modification

1. Introduction

Firmness loss during fruit ripening is contributed mainly by the action of a variety of cell wall modifying enzymes and proteins (Rose and Bennett, 1999; Cosgrove, 2001). Amongst the wall polysaccharides, pectin modification as characterized by increased solubility and depolymerization have been widely studied in fruits such as tomato (Huber, 1983), muskmelon (McCollum et al., 1989), kiwifruit (Redgwell et al., 1992), melon (Rose et al., 1998), papaya (Ali et al., 1998) and carambola (Chin et al., 1999). Apart from pectic polysaccharides, modification of hemicelluloses may also contribute to softening-related wall disassembly during ripening (Knee, 1973; Huber, 1983; McCollum et al., 1989; Ali et al., 1998; Rose et al., 1998; Chin et al., 1999).

Evidence indicates that polygalacturonase (PG) may not be the key enzyme involved in cell wall disassembly even in tomato fruits that are known to contain high levels of *endo*-PG activities (Smith et al., 1988; Giovannoni et al., 1992). In tomato as well as in many other fruits, substantial pectin modifications can proceed in the apparent absence of any significant *endo*-PG activities (McCollum et al., 1989; Giovannoni et al., 1992; Cheng and Huber, 1996). Furthermore, in tropical fruits

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doi:10.1016/j.phytochem.2004.11.005

including carambola that differ markedly in their firmness loss rates, the polygalacturonase activities were attributed predominantly to *exo*-PG (Ali et al., 2004a). Thus, PG alone might not be pivotal to wall modifications and fruit softening. Other wall modifying enzymes, notably pectin methylesterase, *endo*-(1 → 4)β-glucanase, xyloglucan endotransglucosylase/hydrolase and β-galactosidase/galactanase, might be involved in affecting an overall wall disassembly and firmness loss during ripening (Rose and Bennett, 1999; Ali et al., 2004a).

Ripening associated β-galactosidases have been purified and partially characterized from fruits such as tomato (Pressey, 1983; Carey et al., 1995), apple (Dick et al., 1990; Ross et al., 1994), muskmelon (Ranwala et al., 1992), avocado (deVeau et al., 1993), kiwifruit (Ross et al., 1993), persimmon (Kang et al., 1994), mango (Ali et al., 1995), Japanese pear (Kitagawa et al., 1995) and papaya (Ali et al., 1998). β-Galactosidases from these fruit sources occur in multi-forms, seemingly encoded by multi-gene families; however, not all members of the gene family were ripening related (Smith and Gross, 2000; Trainotti et al., 2001). Evidence indicates that some of the major β-galactosidase isoforms are capable to modify pectins that were either in already-soluble forms (Ranwala et al., 1992; deVeau et al., 1993) or in still structurally attached forms (Carrington and Pressey, 1996), apart from ability to also modify alkali-soluble hemicelluloses (Ali et al., 1998; Soh, 2002). The mechanism by which β-galactosidase affects modification of both pectin and hemicellulose is unclear.

In carambola (*Averrhoa carambola* L., family Oxalidaceae) – a non-climacteric (O'Hare, 1997) and slow-softening economically important tropical fruit (Ali et al., 2004a) – ripening related pectin and hemicellulose modifications were accompanied by increased activity of a number of wall degrading enzymes including pectin methylesterase and β-galactosidase (Chin et al., 1999; Ali et al., 2004a). Moreover, accelerated firmness loss during subsequent ripening of chilled-injured carambola at ambient temperature correlated positively with an enhanced increase in activity of both enzymes (Ali et al., 2004b). Thus, it seems, pectin methylesterase and β-galactosidase might contribute significantly to wall modifications during ripening of the fruit. In the present paper, we report the purification and characterization of a β-galactosidase isoform from ripe carambola fruit that appeared to have the capability to markedly modify both pectins and hemicelluloses.

2. Results and discussion

2.1. Purification of β-galactosidase

Initial studies to determine the appropriate amount of extraction buffer to fresh tissue ratio as well as the effects of using ammonium sulphate precipitation as the first purification step were carried out before attempting further purification protocols. It was found that a 2:1 ratio being fresh tissue weight (~1 kg) to extraction buffer volume was suitable and able to retain almost 95% of β-galactosidase activity in the supernatant as well as yielding a total workable extraction volume of about 1.5 l. Increasing the ratio to 3:1 in order to decrease the extraction volume, however, reduced the extractability of the enzyme in the supernatant to only 77%. Ammonium sulphate precipitation of the crude extract followed by dialysis was attempted as an initial purification step to remove other protein as well as to reduce sample volume for subsequent chromatographic procedures. Almost 90% of the β-galactosidase activity was loss at this step. As an alternative, the crude extract was instead dialysed extensively. Dialysis appeared to retain about 75% of the total β-galactosidase activity in the crude extract. Significant loss of activity at this stage might be due to instability of the crude enzyme caused by long standing during dialysis. This step was however essential for sample to be fractionated using ion exchange chromatography.

Typical results for separation and purification of β-galactosidase isoforms are presented in Fig. 1 and summarized in Table 1. Supernatant extracted from 1.2 kg of ripe carambola tissues comprising 10.8 μkat of total β-galactosidase was dialysed and then purified through a series of cation exchange (CM Sepharose fast flow), anion exchange (DEAE-Sepharose fast flow) and gel filtration (Sephacryl S-200) chromatography. Four isoforms of β-galactosidase were resolved. Fractionation of crude extract from ripe fruits on a CM-Sepharose column at pH 5.0 (Fig. 1(a)) was able to remove significant amount of unbound protein, as well as to separate two peaks of β-galactosidase activity (peaks A and B) bound to the column, with stepwise elution using 0.05 and 0.5 M NaCl. One of the peak (peak A) was resolved into two activity peaks – the unbound β-galactosidase I (peak AI) and bound β-galactosidase II (peak AII) – when passed through a DEAE-Sepharose column at pH 7.2 (Fig. 1(b)). The second peak (peak B) was also resolved into two activity peaks, comprising the unbound β-galactosidase III (peak BI) and bound β-galactosidase IV (peak BII) on the same anion exchange column (Fig. 1(c)). Each of the β-galactosidase I and β-galactosidase III peak was fractionated further by gel filtration chromatography to remove contaminating proteins, and both were individually eluted as a single peak through a Sephacryl S-200 HR column (Fig. 1(d) and (e)). In all cases, although significant amount of other proteins were successfully separated from the β-galactosidase isoforms, only the β-galactosidase I peak was particularly enriched in specific activity (Table 1 and Fig. 1). This predominant isoform was purified up to 95-fold (specific activity 5.5 μkat mg^{-1}) with a recov-

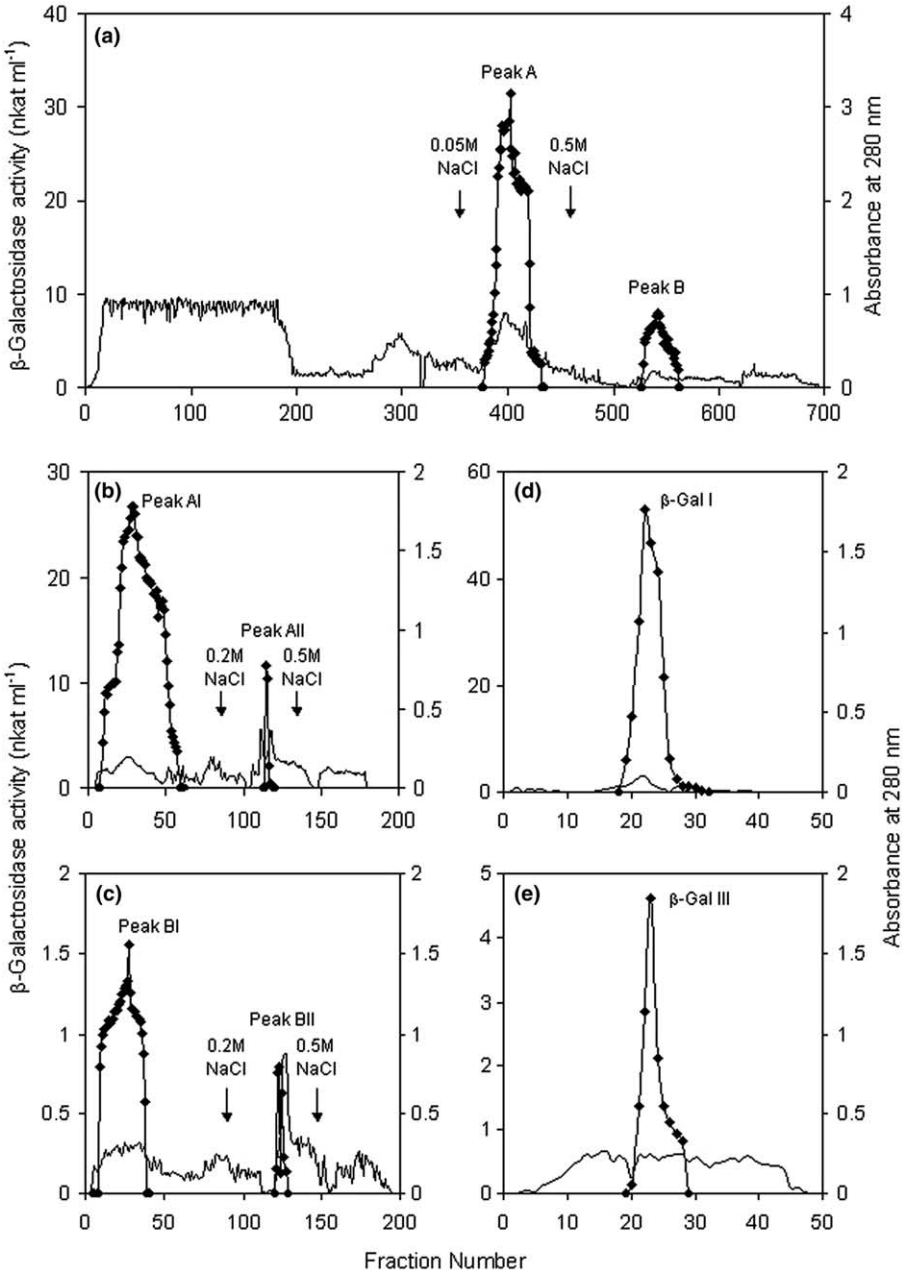

Fig. 1. Elution profile of carambola β-galactosidase activity (●) and absorbance at 280 nm (—) on (a) CM-Sepharose fast flow, (b) DEAE-Sepharose fast flow for peak A, (c) DEAE-Sepharose fast flow for peak B, (d) Sephacryl S-200 for peak AI (β-galactosidase I) and (e) Sephacryl S-200 for peak BI (β-galactosidase III). A fraction (6.2 ml) containing highest specific activity and constituting part of the activity peak of β-galactosidase I as shown in (d), was used in the in vitro wall modification studies.

ery of 12% from the extractable activity. In contrast, isoforms II, III and IV were only purified up to 0.9-, 1.90- and 0.06-fold, respectively (Table 1).

Analysis on native polyacrylamide gels showed that β-galactosidase I migrated as a single protein band, suggesting that it has been purified to homogeneity (Fig. 2(a)). Activities of the potentially significant wall degrading enzymes, polygalacturonase and pectin methylesterase, assayed by the methods described in Lazan et al. (1995), were not detectable in the purified β-galactosidase I fractions. The other isoforms were only partially purified as a number of protein bands were detected on the gel (data not shown). Further purification could not be carried out for these isoforms due to the low levels of protein as well as activity in the fractions.

2.2. Properties of β-galactosidase

The apparent native molecular sizes of β-galactosidase I, II, III and IV estimated by chromatography on

Table 1
Purification of β-galactosidase from ripe carambola

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	186	10813	58	100	1.0
Dialysis	173	7969	46	74	0.8
CM-Sepharose					
Peak A	26	6287	242	58	4.2
Peak B	36	1494	42	14	0.7
DEAE-Sepharose					
Peak AI (β-Gal I)	3.6	5171	1436	48	25
Peak AII (β-Gal II)	3.5	175	50	1.6	0.9
Peak BI (β-Gal III)	3.7	243	66	2.3	1.1
Peak BII (β-Gal IV)	8.3	27	3.3	0.3	0.06
Sephacryl S-200					
β-Gal I	0.24	1328	5533	12	95
β-Gal III	0.82	91	111	0.8	1.9

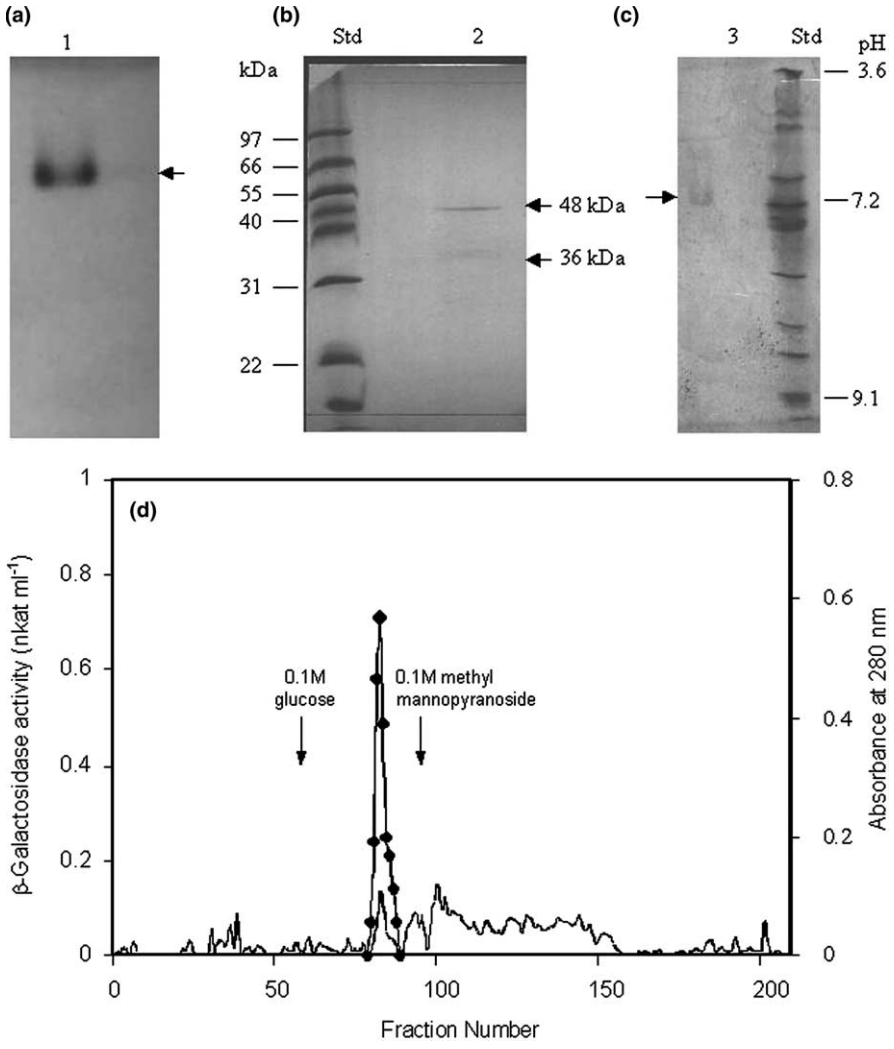

Fig. 2. Fractionation on native (a) and SDS–PAGE (b), and isoelectric focusing (c) of purified β-galactosidase I from ripe carambola fruit, and concanavalin A Sepharose profile of purified β-galactosidase I activity (●) and absorbance (—) at 280 nm (d).

a Sephadex G-200 column were 84, 77, 58 and 130 kDa, respectively. These values were generally comparable with the apparent molecular sizes of β-galactosidase from other fruits such as tomato, 62 kDa (Pressey, 1983), kiwifruit, 60 kDa (Ross et al., 1993), apple, 59 kDa (Ross et al., 1994), pepper, 51 kDa (Biles et al.,

1997) and papaya, 55 and 67 kDa (Ali et al., 1998), whereas the native molecular mass of β-galactosidase IV was comparable with the estimated 118 kDa-size of persimmon fruit β-galactosidase (Kang et al., 1994).

Purified β-galactosidase I as analysed by SDS–PAGE, appeared to comprise two polypeptides with the size of 48 and 36 kDa, suggesting that the native protein is an aggregate of two non-identical subunits (Fig. 2(b)). Presence of two non-identical subunits of β-galactosidase was also observed in persimmon fruit (Kang et al., 1994) and mung bean seedlings (Li et al., 2001), whilst the existence of several polypeptides of β-galactosidase – some of the enzymes appeared to be dimers – has been shown in other fruits such as apples, tomato, Japanese pear and papaya (Ross et al., 1994; Carey et al., 1995; Kitagawa et al., 1995; Ali et al., 1998). Carambola β-galactosidase I was shown to have a glycoprotein property as it was bound to an affinity concanavalin A Sepharose column and can be eluted with 0.1 M glucose (Fig. 2(d)). The possibility that some β-galactosidase proteins might be glycosylated had been reported in mango and papaya as well as in mung bean seedling (Ali et al., 1995, 1998; Li et al., 2001).

As determined by isoelectric focusing, native β-galactosidase I was shown to have a p*I* of 7.2 (Fig. 2(c)). p*I* values in the neutral or basic pH range had also been observed in β-galactosidase from other fruits such as kiwifruit, tomatoes, red pepper and papaya (Ross et al., 1993; Carey et al., 1995; Biles et al., 1997; Ng, 1998), suggesting that certain β-galactosidase isoforms may bind ionically to the acidic cell wall. A number of β-galactosidase isoforms, however, appeared to have p*I* values in the acidic ranges such as those from avocado, mature green pepper and papaya (deVeau et al., 1993; Biles et al., 1997; Ng, 1998).

Analysis of the first 30 N-terminal amino acid sequence of the two subunits of β-galactosidase I was presented in Fig. 3. BLAST searches of the 48-kDa polypeptide indicated that its N-terminal amino acid sequence shared high similarities with the deduced amino acid sequence of many of the published plant β-galactosidases, such as those of strawberry (Accession No. AJ278704, 82%), *Arabidopsis thaliana* (AJ270304, 80%), tomato (AF154423, 80%), mango (AF004812, 80%), papaya (AF064786, 80%), sand pear (AB046543, 80%), chick pea (AJ011010, 76%) and hot chilli (AY029226, 76%) fruits (Fig. 3(a)). The 36-kDa polypeptide showed significant similarity only with a limited number of β-galactosidases, particularly that of the putative strawberry (AJ278704, 85%) protein (Fig. 3(b)). The N-terminal amino acid sequence of the two subunits, however, differed significantly, exhibiting about 20% similarity (Fig. 3(c)). This low degree of similarity between the component polypeptides of β-galactosidase I was ascribed to the fact that significant homologies between the 48-kDa subunit and that of the known β-galactosidases occurred near the N-terminus, whereas for the 36-kDa subunit, homologies occurred near the middle of the proteins sequence (Fig. 3(a) and (b)). It is unclear as whether the polypeptides that constitute the functional β-galactosidase I protein are encoded by different genes or as evidence of high sequence similarity with the strawberry protein seems to suggest, by a single gene (Fig. 3(a) and (b)). Overall, our results imply that the putative products of the carambola β-galactosidase I gene/genes may have to undergo certain modifications either at the post-transcriptional or post-translational stages, as had been reported for persimmon β-galactosidase (Kang et al., 1994). Plant β-galactosidases may contain more than one glycosylation domains (Trainotti et al., 2001), and in the case of the β-galactosidase I, one of the potential *N*-glycosylation sites seemed to reside within the larger-size subunit (Fig. 3). This result corroborates the evidence that β-galactosidase I was possibly a glycoprotein (Fig. 2(d)).

The apparent K_m values for β-galactosidase I, II, III and IV against *p*-nitrophenyl β-D-galactopyranoside (PNPG) estimated from Lineweaver–Burk plots were 21.7, 4.2, 3.3 and 2.0 mM, respectively. The V_{max} values for the respective isoforms were 5.7, 31.6, 33.6 and 5.5 nkat mg^{-1} protein. All isoforms lost 50% of its enzymatic activity at 65 °C, comparable with the results reported for tomato (Pressey, 1983), mango (Ali et al., 1995), and papaya (Ali et al., 1998). Isoforms of β-galactosidase were optimally active at acidic pH ranging from pH 3.0–4.0. β-Galactosidases isolated from muskmelon, kiwifruit and papaya also seemed to be optimally active at acidic pH range (Ranwala et al., 1992; Ross et al., 1993; Ali et al., 1998).

Specificity studies of β-galactosidase isoforms towards some of the synthetic and endogenous substrates showed that the purified β-galactosidase I, and the semi-purified β-galactosidase II and III had no α-galactosidase, α-arabinosidase, β-glucosidase and α-mannosidase activity, and none of the four isoforms was able to degrade birchwood xylan. β-Galactosidase I was found to be substantially active in hydrolyzing the endogenous wall polymers (1 → 4)β-linked spruce galactan at a rate twice as rapid as that against the (1 → 3)β- and (1 → 6)β-linked gum arabic galactan. To a limited extent, the enzyme also was capable to degrade arabinogalactan. Purified β-galactosidases from ripe mango and papaya fruits were also capable to hydrolyse both spruce and gum arabic galactans (Ali et al., 1995, 1998). However, unlike the isoform I of carambola, all the three β-galactosidases of papaya showed significantly higher activity against galactan with the (1 → 3)β- and (1 → 6)β-linkages than with the (1 → 4)β-linkages (Ali et al., 1998). Since galactans and arabinogalactans might exist as rhamnogalacturonan 1 (RG1) side-chains or as glycan cross-links that inter-connect RG1 and xyloglucan-cellulose microfibrils networks (Keegstra et al.,

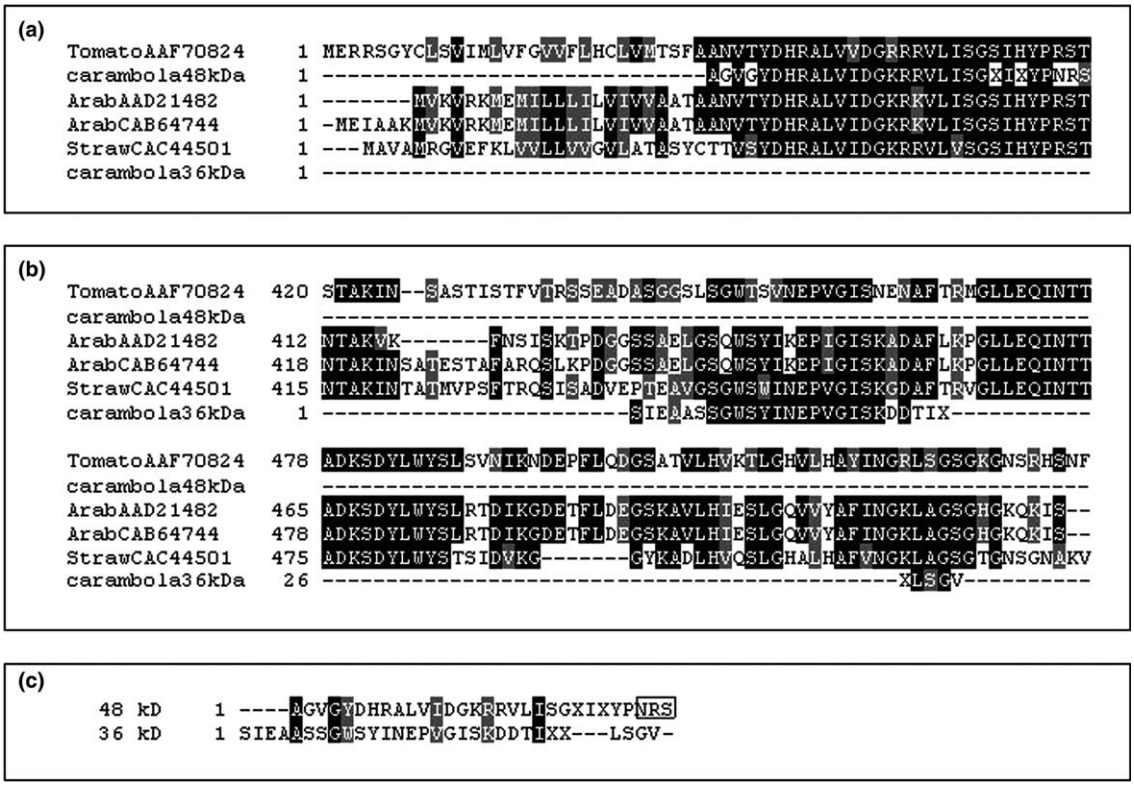

Fig. 3. Alignment of the N-terminal amino acid sequence of the (a) 48-kDa and (b) 36-kDa subunits of carambola β-galactosidase I with the deduced amino acid sequence of selected plant β-galactosidases, and (c) sequence alignment of the two subunits of β-galactosidase I. Accession numbers for the selected strawberry CAC44501, *Arabidopsis thaliana* CAB64744 and AAD21482, and tomato AAF70824 β-galactosidases are AJ278704, AJ270304, A006587 and AF154423, respectively. Identical amino acid residues and conservative substitutions are shaded as dark and grey areas, respectively. Putative Asn glycosylation site (N–X–S) that reside in the 48-kDa-polypeptide sequence is indicated in (c).

1973; Carpita and McCaan, 2000), thus, functional capacity as β-galactanases probably suggests that the carambola enzyme have the potential to modify the cell wall.

2.3. Significance of β-galactosidase I to carambola fruit softening

In order to evaluate the functional significance of β-galactosidase I as a wall-modifying enzyme, in vitro wall modification studies were carried out as a comparison to changes that occur during ripening. As ripening progressed and fruit firmness decreased, pectins as reflected by changes in the levels of chelator-soluble polyuronides (CSP) and carbohydrates (CSC), were increasingly solubilized (Table 2) and depolymerized (Fig. 4(a) and (b)). Likewise, alkaline-soluble hemicelluloses were depolymerized with ripening (Fig. 4(c)). Results of incubating the purified β-galactosidase I with unripe fruit's CW preparation that contains pectins that are still structurally attached to the wall network or with the hemicellulose fractions extractable in alkali showed that the isoform was capable of solubilizing (Table 2) as well as downsizing the pectins (4d and 4e) and the hemicelluloses (Fig. 4(f)). The extent of the modifications appeared comparable with the changes that occur *in situ* during normal ripening, thus, suggesting the potential significance of this isoform in softening-related wall dis-

Table 2
Changes in tissue firmness (Newton) and levels of chelator-soluble polyuronides (CSP) and carbohydrates (CSC) in ripening carambola fruit and in vitro pectin solubilisation from unripe fruit's cell walls by purified β-galactosidase I after 22 h incubation

Sample	Texture	CSP (N) (μg/mg EIR)	CSC (μg/mg EIR)
Unripe fruit (day 0)	35 ± 3	43 ± 0.4	47 ± 0.3
Half ripe fruit (day 12)	21 ± 2	66 ± 0.7	72 ± 0.6
Ripe fruit (day 24)	9 ± 0.2	83 ± 0.1	100 ± 0.7
Unripe tissue + boiled β-galactosidase I	–	41 ± 0.4	50 ± 0.4
Unripe tissue + active β-galactosidase I	–	98 ± 0.4	122 ± 2.9

Values are means of 4 fruits or analyses ±SE.

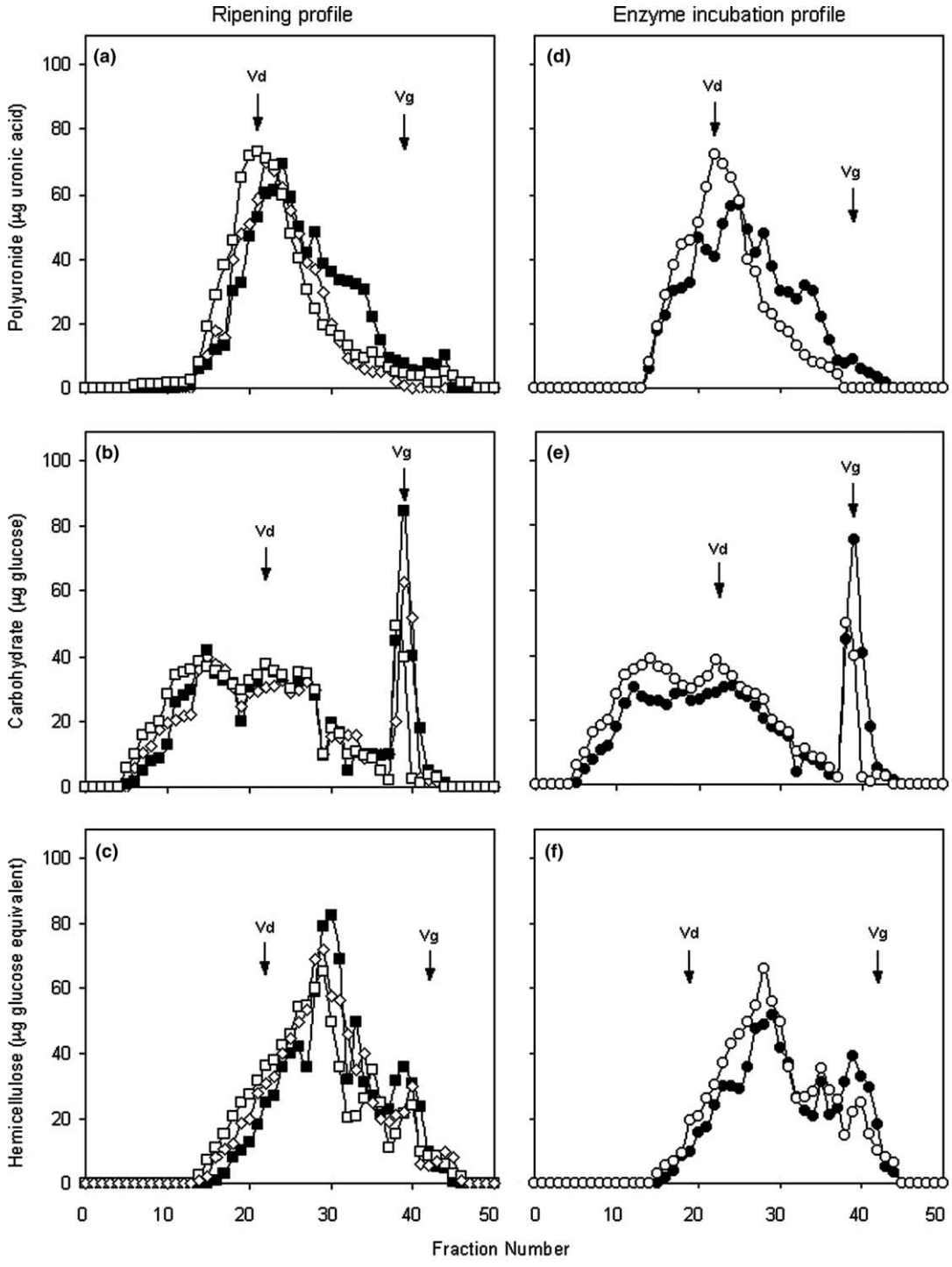

Fig. 4. Sephacryl S-500 profiles of EDTA-soluble polyuronides and carbohydrates of ripening carambola (a) and (b) and of unripe fruit's cell wall upon incubation with purified β-galactosidase I for 22 h (d) and (e), and the profiles for 4 M alkali-soluble hemicellulose of ripening carambola (c) and of unripe fruit's hemicellulose fraction upon incubation with the purified enzyme (f). Elution volume for dextran blue 2000 (V_d) and glucose (V_g) was shown. Ripening stage, day 0 (□), day 12 (◇) and day 24 (■), and active enzyme (●), boiled enzyme (○).

assembly during ripening (Table 2 and Fig. 4; Chin et al., 1999). Besides carambola, β-galactosidases from ripening papaya were also capable to modify both the structurally attached pectins as well as the alkaline-soluble hemicelluloses (Ali et al., 1998; Soh, 2002). The mechanism by which β-galactosidase with β-galactanase activity can substantially affect modification of the wall polysaccharides is unclear and need further investigation. Ability of β-galactosidases to hydrolyse synthetic PNPG substrate suggests that they might have an *exo*-acting activity. However, the ability of the carambola enzyme to significantly affect modification of a more

complex and structurally intact cell wall components could not discount the possibility that the enzyme might also function as an *endo*-glycanase.

3. Experimental

3.1. Plant material

Carambola fruit (*A. carambola* L. cv. B10) of uniform size were harvested at commercial maturity stage (Day 0: stage 2, light green) from a private farm in Semenyih, Selangor, Malaysia. Fruits were rinsed with water, soaked for 5 min in 0.02% banlate, air-dried and left to ripen (Day 24: stage 6, orange) at ambient temperature. The ripe fruits were peeled, cut into 1 cm^3 cubes, frozen in liquid nitrogen and then stored at −80 °C until required.

3.2. Purification of β-galactosidase

Purification was carried out at 4 °C. Enzyme was extracted according to the methods described in Chin et al. (1999). About 1.2 kg of frozen tissue was blended to fine powder and homogenized in 2:1 ratio of cold extraction buffer (0.1 M sodium citrate, pH 4.6, containing 1 M NaCl, 13 mM EDTA, 10 mM β-mercaptoethanol and 1% (w/v) soluble polyvinylpyrrolidone (PVP-40)) using a blender (Edmund Buhler 7400, Tübingen, Germany). The homogenate was kept on ice for 30 min with occasional stirring before centrifuging at 17,000*g* (Sorvall Superspeed RC-5B, SS34 rotor) for 30 min. The supernatant was filtered through two layers of nylon cloth. The filtrate was then dialyzed overnight against 0.01 M acetate buffer, pH 5.0, containing 10 mM β-mercaptoethanol with twice changes of fresh buffer. Following dialysis, the sample was loaded onto a CM-Sepharose fast flow (Pharmacia) column (5.0 cm × 10.0 cm), previously equilibrated with the same acetate buffer containing 1 mM DTT. Fractions (8.0 ml) were collected at a flow rate of 75 ml h^{-1}. No β-galactosidase activity was detected in the unbound fractions. Bound proteins were further eluted stepwisely using 0.05 and 0.5 M NaCl. β-Galactosidase activity was detected in both the 0.05 M NaCl (peak A) and 0.5 M NaCl eluents (peak B).

Peaks A and B were pooled separately and dialysed overnight against 0.01 M Tris–HCl, pH 7.2, containing 10 mM β-mercaptoethanol. The respective peaks were then loaded separately onto a DEAE-Sepharose fast flow (Pharmacia) column (2.5 cm × 15.0 cm) previously equilibrated with the same buffer containing 1 mM DTT. Fractions (7.2 ml) were collected at a flow rate of 55 ml h^{-1}. From peak A, two peaks having β-galactosidase activity were recovered namely, peak AI, obtained in the unbound fraction, and peak AII (β-galactosidase II), eluted in 0.2 M NaCl. Proteins in the pooled fractions of peak AI were precipitated with 85% ammonium sulphate. Pellet was redissolved in 0.05 M Tris–HCL, pH 7.2, containing 1 mM DTT and then passed through a Sephacryl S-200 HR (Pharmacia) column (2.5 cm × 40 cm), previously equilibrated with the same buffer. Fractions of 6.2 ml were collected at a flow rate of 40 ml h^{-1}. One prominent activity peak, designated as β-galactosidase I, with a corresponding single protein peak was obtained.

Two β-galactosidase activity peaks were also obtained from peak B, namely peak BI and peak BII (β-galactosidase IV) from the DEAE-Sepharose column. Fractions from peak BI having high specific activity were pooled and precipitated with 85% ammonium sulphate. Pellet was dissolved in 0.05 M Tris–HCl, pH 7.2, containing 1 mM DTT and loaded onto a Sephacryl S-200 HR (Pharmacia) column (2.5 cm × 40 cm), previously equilibrated with the same buffer. A prominent β-galactosidase activity peak (β-galactosidase III) was recovered. Peak fractions of β-galactosidase I, II, III and IV with high specific activity were used in physical characterization and kinetic studies of the enzymes, whilst a fraction with the highest specific activity and exhibiting only β-galactosidase I band on native gel was used in in vitro cell wall modification studies.

3.3. Assay of β-galactosidase

β-Galactosidase was assayed according to the methods described in Lazan et al. (1995). The assay mixture consisted of 0.52 ml 0.1 M sodium citrate, pH 4.1, 0.4 ml 0.1% (w/v) BSA and 0.4 ml 13 mM *p*-nitrophenyl-β-D-galactopyranoside (PNPG) as substrate. The reaction mixture was preincubated at 37 °C for 10 min before addition of 0.08 ml enzyme. The reaction was further incubated for 15 min before addition of 2 ml 0.2 M Na_2CO_3. Boiled enzyme was used as a control in the assay mixture. The amount of *p*-nitrophenol formed was determined spectrophotometrically through absorbance at 415 nm. Enzyme activity was expressed as nmol *p*-nitrophenol formed per second (nkat).

3.4. Protein determination

Samples from crude extracts were precipitated with an equal volume of 10% (w/v) TCA, rinsed twice with 5% TCA and redissolved in 0.1 M NaOH prior to Bradford (1976) assay, using bovine serum albumin (BSA) as a standard. Protein content of chromatographic fractions was estimated from the absorbance at 280 nm.

3.5. Molecular mass estimation

The apparent molecular mass of native β-galactosidase protein was estimated using a Sephadex G-200

(Pharmacia) column (2.5 cm × 40 cm) calibrated with 5 mg ml^{-1} of ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa) and catalase (232 kDa) and 1 mg ml^{-1} of blue dextran (200 kDa) [Sigma High Molecular weight Calibration kit]. The column was equilibrated with 50 mM potassium phosphate, pH 7.0, containing 10 M NaCl and 0.02% (w/v) sodium azide and eluted at a flow rate of 26 ml h^{-1}.

3.6. Gel electrophoresis

Polyacrylamide gel electrophoresis was done according to the methods of Laemmli (1970) for SDS–PAGE and Reisfeld et al. (1962) for native-PAGE using 12% and 15% acrylamide, respectively. About 2 μg of β-galactosidase protein was loaded for native-PAGE whereas 1.5 μg for SDS–PAGE. Analytical isoelectric focusing was performed using precast Ampholine PAG plates (Pharmacia, LKB, Uppsala, Sweden) and stained with Coomassie brilliant blue R-250 exactly according to the manufacturer's instructions.

3.7. N-terminal sequencing

Amino acid sequencing of 48 and 36 kDa subunits of β-galactosidase I was performed as described in Matsudaira (1987). About 20 μg of purified β-galactosidase I protein was run on SDS–PAGE and the resolved proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA) in 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid buffer containing 10% methanol (pH 11). The PVDF membrane was stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol, destained in acetic acid and methanol (1:5) mixture, rinsed in deionized water and then air-dried. Protein bands were excised and submitted to Microchemical Facility, The Babraham Institute of Animal Physiology and Genetics, Cambridge, UK for N-terminal sequencing. Sequence comparison and alignment were performed using the BLAST searches (NCBI) and BCM Search Launcher respectively.

3.8. Glycoprotein property

β-Galactosidase I was ascertained for its glycoprotein properties using a Con-A Sepharose (Pharmacia) column (1.5 cm × 3.0 cm). Purified β-galactosidase I fractions from Sephacryl S-200 column was dialysed extensively against 0.02 M Tris–HCl, pH 7.4, containing 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ before being loaded onto the affinity column previously equilibrated with the same buffer, and fractions were collected at a flow rate of 55 ml h^{-1}. Bound proteins were eluted with buffer containing 0.1 M glucose or 0.1 M methylmannopyranoside, and β-galactosidase activity and absorbance at 280 nm were monitored.

3.9. Kinetics and substrate specificity studies

Values of K_m and V_{max}, optimal pH and temperature, heat stability as well as studies on the ability of isolated β-galactosidase isoforms to hydrolysed synthetic and endogenous substrates were done as described in Ali et al. (1998).

3.10. Extraction, analysis and in vitro modification of pectin and hemicellulose by β-galactosidase I

Soluble pectins were extracted from ethanol insoluble residues (EIR) by the methods described in Chin et al. (1999). Briefly, 100 mg of EIR from day 0, 12 or 24 fruit was suspended (37 °C, 8 h) in 10 ml of 30 mM NaOAc, pH 5.0, containing 10 mM EDTA. The supernatant was resolved by centrifugation (Biofuge 17RS Heraeus Sepatech) at 2000*g* for 30 min. Amounts of uronic acid (Blumenkrantz and Asboe-Hansen, 1973) and carbohydrate (Dubois et al., 1956) were estimated.

Cell wall preparation from day 0, 12 or 24 fruit was done according to the protocols as described earlier (Chin et al., 1999). For hemicellulose extraction, about 500 mg of the wall material was incubated for 8 h in 7 ml 4 M NaOH containing 0.24 M $NaBH_4$. This procedure was repeated after filtration, and the combined filtrate was neutralized with concentrated acetic acid. The extracts were then dialysed against tap water for 12 h followed by 10% methanol and distilled water for 24 h, respectively. The amount of extractable hemicellulose (as glucose equivalent) was determined (Dubois et al., 1956).

For analysis of ripening-related pectin depolymerization, about 800 μg CSP was loaded onto a Sephacryl S-500 column (1.6 cm × 50 cm) previously equilibrated with 80 mM NaOAc, pH 5.0, containing 10 mM EDTA and 50 mM NaCl. Fractions (1.8 ml) were collected at a flow rate of 20 ml h^{-1} and levels of uronic acid and carbohydrate were determined. For hemicellulose depolymerization analysis, about 800 μg of alkaline-soluble hemicellulose was loaded onto a Sephacryl S-500 (1.6 cm x 45.0 cm) column previously equilibrated with 15 mM sodium citrate phosphate pH 5.5, containing 100 mM NaCl, and 2 ml fractions were collected at a flow rate of 20 ml h^{-1}. The levels of hemicellulose in each fraction were determined (Dubois et al., 1956).

Modification of pectins and hemicelluloses by purified β-galactosidase I was analysed by the protocols described in Ali et al. (1998). For pectin, about 15 mg EIR from unripe (stage 2) fruit was incubated (37 °C, 22 h) with about 6 nkat purified β-galactosidase I, 5 ml 30 mM sodium acetate, pH 5.0, containing 10 mM EDTA and 2 drops of toluene. After stopping the reaction by

boiling (5 min), the supernatant was recovered by centrifugation (MSE superspeed at 2000*g* for 30 min). Levels of uronic acid and carbohydrate were determined, and gel filtration analysis was performed as described above. For hemicellulose modification, about 800 μg of the unripe fruit alkaline-soluble hemicellulose in 2.4 ml buffer was incubated (37 °C, 22 h) with about 6 nkat of the purified enzyme in the presence of 1–2 drops of toluene and the reaction was stopped by boiling (5 min). Hemicellulose depolymerization was analysed using a Sephacryl S-500 column as described above. Boiled β-galactosidase I was used as controls in this in vitro wall modification studies.

Acknowledgement

We thank the Ministry of Science, Technology and Environment of Malaysia for their financial support (IRPA Project 01-02-02-0036).

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